Acta Physiol Plant (2014) 36:903–913
DOI 10.1007/s11738-013-1469-5

ORIGINAL PAPER

Copper phytoextraction with *Salix purpurea* × *viminalis* under various Ca/Mg ratios. Part 2. Effect on organic acid, phenolics and salicylic acid contents

Kinga Drzewiecka · Mirosław Mleczek · Monika Gąsecka · Zuzanna Magdziak · Piotr Goliński · Tamara Chadzinikolau

Received: 6 April 2013 / Revised: 18 November 2013 / Accepted: 16 December 2013 / Published online: 29 December 2013

Abstract One-year-old cuttings of basket willow (*Salix purpurea* × *viminalis*) were cultivated hydroponically under increasing Cu concentrations (0, 1, 2 and 3 mM) and at four Ca/Mg ratios (4:1, 1:10, 20:1 and 1:$^1/_4$). After 14 days, rhizosphere and leaf samples were analysed. *Salix* plants were able to release relatively high amounts of low molecular weight organic acids (LMWOAs) in a short period of time. The total amount of LMWOAs increased with increasing Cu concentrations. Oxalic and acetic acids were dominant, and act as complexing agents for Cu ions, and therefore, organic exudates should be taken into account in phytoextraction of polluted areas. The Ca/Mg ratio of the medium significantly influenced not only concentration, but also the composition of LMWOAs. Phenolics content in leaves increased with the excess of Ca and Mg and with Cu level in the medium for all Ca/Mg ratios. The accumulation of glucose, fructose and sucrose in leaves was observed for deficiency and excess of Ca and/or Mg and Cu treatment at all Ca/Mg ratios. Excess calcium (Ca/Mg = 20:1) led to strong induction of salicylic acid biosynthesis, probably resulting from enhanced oxidative stress.

Keywords Ca/Mg ratio · Copper · Oxidative stress · *Salix Salix purpurea* × *viminalis*

Communicated by E. Kuzniak-Gebarowska.

Electronic supplementary material The online version of this article (doi:10.1007/s11738-013-1469-5) contains supplementary material, which is available to authorized users.

K. Drzewiecka · M. Mleczek (✉) · M. Gąsecka · Z. Magdziak · P. Goliński
Department of Chemistry, Poznan University of Life Sciences, Wojska Polskiego 75, 60-625 Poznan, Poland
e-mail: mirekmm@up.poznan.pl

T. Chadzinikolau
Department of Plant Physiology, Poznan University of Life Sciences, Wolynska 55, 60-637 Poznan, Poland

Introduction

Contamination with heavy metals is one of the prime concerns in protection of the environment. Heavy metals in low concentrations are necessary in metabolic pathways (usually they are enzyme promoters), but also they are toxic to humans, animals and plants in small or insignificantly higher concentrations. Conventional clean-up technologies are expensive, difficult and time-consuming, and they destroy the structure of the soil (i.e. texture and organic matter) (Strobel 2001). Phytoremediation with hyperaccumulators is an alternative method to removal of inorganic pollutants—especially heavy metals—from soil with no negative changes in soil structure and is employed to absorb, accumulate and to transport metals from soil to the harvestable parts from metal polluted soil (Najeeb et al. 2009).

More recent studies on phytoextraction have focused on fast-growing plants, especially tree and bush species such as selected willows and poplars taxa. Although these species are not recognized as hyperaccumulators, their properties combine heavy metal accumulation with high biomass yields, and they can be employed in recultivation of heavily contaminated sites (Lewandowski et al. 2006). Moreover, the use of tree species has several advantages, which include deeper root system, high productivity of biomass and high transpiration system activity. The high biomass production

of willow species has been widely reported (Tlustŏs et al. 2007; Stolarski et al. 2008). Moreover, *Salix* sp. easily adapts to new environmental conditions and undergoes changes permitting high tolerance of weather conditions (Hermle et al. 2006). Furthermore, *Salix* species have been widely used for biomass production (energy plant), as well as in the pulp and paper industry and in factories producing particle board. However, the potential for use of willow (e.g. basket willow, *Salix viminalis* L.) in phytoremediation of metals or in recultivation of polluted soils is affected by numerous factors, such as cation exchange capacity, pH values of the soil, excess amounts of fertilizers, chelators, composition and amount of heavy metals and the influence of Ca/Mg ratio in soil, and depends on the resulting toxicity of the metal to plant species/taxon (Parker et al. 1998; Ösretås and Greger 2006). Among these factors, the Ca/Mg ratio can be easily modified prior to remediation/recultivation to enhance plant resistance and to deplete the level of oxidative stress caused by metallic ions.

Calcium and magnesium are macroelements necessary for plant growth. Ca influences the uptake and transport of other elements essential for plants (White and Broadley 2003), and regulates hydration and regular cell structure (Scrase-Field and Knight 2003). Mg is essential for chlorophyll biosynthesis, and its deficiency causes serious disturbances in photosynthesis resulting in deterioration of growth, development and yield of plants (Kabata-Pendias and Pendias 1999). It has been hypothesized that plants selectively control the amount and form of Ca, Mg and other metals and additionally, distribution of them from roots to the aboveground tissues (Oze et al. 2008). Copper is an important micronutrient for plants and is used as a redox cofactor in numerous physiological processes, including photosynthesis, mitochondrial respiration, superoxide scavenging, ethylene perception, etc. Besides being essential, copper is toxic when present in excess in soil, water, and the atmosphere, and has become a global environmental problem because of mining, industrial, and agricultural practices (Elguindi et al. 2011). The reactivity of Cu ions can lead to the generation of harmful reactive oxygen species via Fenton and Haber–Weiss reactions that cause severe oxidative damage to cells (Peñarrubia et al. 2010), disturbances in plant metabolism (Ait Ali et al. 2004), low seed set and leaf chlorosis (Albarracín et al. 2010).

The objective of this study was to determine the influence of Ca^{2+} and Mg^{2+} concentrations and their molar ratio on the *Salix purpurea* × *viminalis* response to elevated copper concentrations in the cultivation medium.

Materials and methods

Plant material

One-year-old cuttings of *Salix purpurea* × *viminalis* (the older name before botanical verification was *Salix viminalis* L. cv. 'Cannabina') collected from 3-year-old rootstock without foliage were used in the experiment. To induce root formation, standardized rods (25 cm long, 15 mm in diameter) were incubated in modified Knop's medium (Barabasz et al. 2010). The characteristics of modified Knop's medium composition are presented in Table 1.

Nutrient solution was adjusted to pH 5.8 (PN-ISO 10390:1997) and electrolytic conduction (PN-ISO 1265 + AC1:1997) of nutrient solution was 1.63 mS cm^{-1}.

Concentrations of the most significant ions were as follows: 4.23 mM of Ca^{2+}, 1.04 mM of Mg^{2+}, 6.63 mM of NO_3^-, 1.84 mM of PO_4^{3-}, 4.32 mM of K^+, and 1.06 mM of SO_4^{2-}. To improve root formation, the solution applied at the beginning of the preliminary incubation period contained 50 % of salt contents in the standard Knop's medium, which also facilitated easier and faster adaptation of plants to the new conditions. After 10 days plants were selected according to the size of the root system to obtain a uniform group and transferred into Knop's medium (0.5 L) containing copper $Cu(NO_3)_2 \times 6H_2O$ salts at 0, 1, 2, 3 mM addition levels stabilized with steamed ultrapure quartz sand in hydroponic pots (one *Salix* cutting was used per pot and three plants per each combination in one of two independent experiments). Ca^{2+} and Mg^{2+}, in the form of

Table 1 Concentration of main components and microelements of modified Knop's medium

Main components	Concentration (mM)
$Ca(NO_3)_2$	4.24
KNO_3	2.5
KH_2PO_4	1.84
$MgSO_4 \cdot 7H_2O$	1
$FeSO_4 \cdot 7H_2O$	0.045
Microelements	**Concentration (μM)**
NaFeEDTA	10
H_3BO_3	6.25
$MnCl_2$	0.5
$ZnSO_4$	0.5
$CuSO_4$	0.025
Na_2MoO_4	0.125
$CoCl_2$	0.025
KJ	1.25

Table 2 Concentration (mM) of Ca and Mg in modified Knop's medium in relation to Ca/Mg ratio

Ca/Mg ratio	Concentration	
	Ca^{2+}	Mg^{2+}
4:1	4.23	1.06
20:1	21.16	1.06
1:10	1.06	10.6
$1:^1/_4$	1.06	0.26

$Ca(NO_3)_2$ and $MgSO_4$, were added with other macroelements of Knop's medium, and Ca/Mg ion ratios were as follows: 4:1—the reference (control), where $C_{Ca} = 4.23$ mM and $C_{Mg} = 1.06$ mM, 1:10 ($C_{Ca} = 1.06$ mM and $C_{Mg} = 10.6$ mM), 20:1 ($C_{Ca} = 21.16$ mM and $C_{Mg} = 1.06$ mM) and $1:^1/_4$ ($C_{Ca} = 1.04$ mM and $C_{Mg} = 0.26$ mM) (Table 2).

The 14-day experiment was performed in a climate chamber under controlled conditions (air temperature 21–15 ± 1 °C, relative humidity 79 ± 1 %), equipped with a fluorescent lamp (TL-D 36 W/865G13 Philips) providing a radiation (photon) flux of 255 μE s^{-1} m^{-2} (μmol s^{-1} m^{-2}) at the top of the plant for 16 h a day.

Selected parameters analysis

Analyses of low molecular weight organic acids (LMWOAs), total phenolics, free salicylic acid and soluble carbohydrates were performed according to methodology described by Drzewiecka et al. (2012a) and additionally, were presented in electronic Supplementary Material.

Thiols measurement

Glutathione (GSH) and phytochelatins (PCs) were isolated and separated according to Stroiński and Zielezińska (1997), based on the Tukendorf and Rauser (1990) method. Frozen leaves (0.5 g) were homogenized with an Art-Miccra homogenizer in 2 mL of 0.5 mM HCl. The GSH and PCs were estimated, using reverse-phase HPLC (Varian Pro Star) with post-column derivatization using Ellman's reagent and measured at $\lambda = 405$ between 22 and 28 min of elution from the column.

Statistical analysis

Statistical analysis was performed with Statistica 8 software provided by StatSoft. The single fixed effects of the two experimental factors, i.e. Cu^{2+} addition level to the cultivation medium and the value of Ca/Mg molar ratio, on selected parameters of *Salix Salix purpurea* × *viminalis* physiology was tested with two-way analysis of variance (ANOVA) at $\alpha = 0.05$ using the F test (F and empirical p values presented in table). Significance of differences between means was tested with post hoc Tukey's HSD test and presented in charts in the form of confidence intervals at a confidence level of 95 %. Concentrations of selected LMWOAs are expressed in the table as mean values with superscripts indicating significant differences for the 'Cu level × Ca/Mg ratio' interaction at $p \leq 0.05$. The correlation between the level of physiological parameters and Cu concentration in the medium was tested with Pearson's correlation coefficient (r).

Table 3 F statistic values and their significance in two-factor analysis of variance (two-way ANOVA) at $\alpha = 0.05$

Biological active compounds	F statistic value in two-way ANOVA		
	Single effect		Fixed effect
	Cu addition level (A)	Ca/Mg ratio (R)	$A \times R$
Total LMWOAs	834***	1,361***	341***
Total phenolics	879***	229***	78***
Salicylic acid	2,512***	1,023***	420***
Glucose	264***	47***	13***
Fructose	2,224***	230***	81***
Sucrose	498***	242***	8***
Glutathione	122***	65***	1.3 ns

ns not significant

*** Significant at $p \leq 0.001$

Results

According to the two-way analysis of variance, all the biological active compounds analysed throughout the present study differentiated significantly ($p \leq 0.001$) *Salix* plants taking into consideration the level of copper addition to the medium, Ca/Mg ratios, as well as the interaction of the two experimental factors (with the exception of glutathione content in willow leaves). However, a stronger effect was observed in the case of Cu level, excluding the total concentration of LMWOAs, which was strongly affected by the Ca/Mg ratio (Table 3).

LMWOA contents

To investigate the composition and concentration of LMWOAs in *Salix viminalis* L. rhizosphere, plants were exposed to increasing amounts of Cu added to the cultivation medium at four different Ca/Mg ratios. Ten LMWOAs were detected (oxalic, citric, malic, malonic, acetic, fumaric, maleic, formic, lactic and succinic acid) for

Table 4 Low molecular weight organic acid contents (μM kg^{-1} DW) in the rhizosphere of Cu-treated *Salix purpurea* × *viminalis* plants cultivated at different Ca/Mg ratios—mean values ± SD ($n = 6$; *nd* not detected)

Cu concentration level (mM L^{-1})	Acetic acid $C_2H_4O_2$	Citric acid $C_6H_8O_7$	Formic acid CH_2O_2	Fumaric acid $C_4H_4O_4$	Lactic acid $C_3H_6O_3$	Maleic acid $C_4H_4O_4$	Malic acid $C_4H_6O_5$	Malonic acid $C_3H_4O_4$	Oxalic acid $H_2C_2O_4$	Succinic acid $C_4H_6O_4$	LMWOAs total of 10 acids
Ca/Mg = 4:1											
0	$2.37^{d} \pm 0.10$	$0.30^{h} \pm 0.02$	$0.24^{g} \pm 0.05$	nd	$0.45^{fg} \pm 0.04$	nd	nd^{c}	$0.25^{b} \pm 0.03$	$34.68^{c} \pm 0.07$	nd	$38.29^{g} \pm 0.10$
1	$15.25^{b} \pm 1.20$	$0.41^{h} \pm 0.05$	$1.60^{d} \pm 0.31$	$0.01^{b} \pm 0.00$	$0.40^{g} \pm 0.03$	nd	$0.15^{i} \pm 0.02$	$0.22^{b} \pm 0.05$	$74.80^{b} \pm 2.28$	$0.71^{e} \pm 0.09$	$93.55^{c} \pm 3.52$
2	$6.35^{c} \pm 4.06$	$1.92^{g} \pm 0.01$	$1.14^{de} \pm 0.12$	$0.01^{b} \pm 0.01$	$0.40^{g} \pm 0.20$	nd	$0.14^{i} \pm 0.10$	$0.14^{bc} \pm 0.04$	$68.39^{b} \pm 0.74$	$0.90^{cd} \pm 0.24$	$87.13^{c} \pm 7.60$
3	$24.30^{a} \pm 0.91$	nd	$1.80^{d} \pm 0.24$	nd	$0.82^{ef} \pm 0.37$	nd	$0.13^{i} \pm 0.02$	$0.67^{a} \pm 0.09$	$192.34^{a} \pm 9.49$	$6.79^{a} \pm 0.72$	$226.85^{a} \pm 10.48$
Ca/Mg = 20:1											
0	$0.78^{f} \pm 0.01$	$16.78^{f} \pm 0.37$	$6.69^{c} \pm 0.14$	nd	$1.54^{d} \pm 0.02$	$0.01^{g} \pm 0.00$	$0.32^{fg} \pm 0.00$	nd	$0.03^{j} \pm 0.00$	$1.74^{b} \pm 0.04$	$27.89^{h} \pm 0.46$
1	$1.12^{e} \pm 0.02$	$19.29^{e} \pm 0.04$	$7.19^{c} \pm 0.20$	nd	$1.14^{e} \pm 0.02$	$0.47^{e} \pm 0.01$	$0.42^{e} \pm 0.01$	$0.16^{bc} \pm 0.01$	$1.03^{h} \pm 0.02$	$0.88^{d} \pm 0.00$	$31.70^{h} \pm 0.22$
2	$1.24^{e} \pm 0.01$	$28.75^{d} \pm 0.53$	$9.19^{b} \pm 0.29$	nd	$1.03^{e} \pm 0.02$	$0.91^{c} \pm 0.03$	$0.42^{e} \pm 0.01$	$0.03^{ed} \pm 0.01$	$0.66^{i} \pm 0.00$	$0.33^{h} \pm 0.00$	$42.56^{g} \pm 0.25$
3	$2.04^{d} \pm 0.04$	$117.15^{a} \pm 0.28$	$12.95^{a} \pm 0.04$	nd	$1.52^{d} \pm 0.01$	$0.97^{b} \pm 0.01$	$0.32^{fg} \pm 0.01$	$0.02^{ed} \pm 0.01$	$0.08^{j} \pm 0.00$	$0.42^{g} \pm 0.00$	$135.47^{b} \pm 0.30$
Ca/Mg = 1:1/4											
0	$0.63^{f} \pm 0.01$	$31.36^{d} \pm 0.35$	$1.02^{e} \pm 0.06$	nd	$0.23^{g} \pm 0.01$	$0.02^{g} \pm 0.01$	$0.41^{e} \pm 0.01$	$0.11^{cd} \pm 0.01$	$6.55^{f} \pm 0.20$	$0.59^{f} \pm 0.01$	$40.92^{g} \pm 0.16$
1	$2.65^{d} \pm 0.11$	$34.49^{cd} \pm 0.21$	$0.60^{f} \pm 0.01$	nd	$0.32^{g} \pm 0.00$	$0.02^{g} \pm 0.01$	$0.42^{e} \pm 0.01$	$0.04^{ed} \pm 0.01$	$17.08^{d} \pm 0.12$	$0.71^{e} \pm 0.01$	$56.33^{e} \pm 0.12$
2	$0.65^{f} \pm 0.00$	$52.29^{b} \pm 0.41$	$1.07^{e} \pm 0.05$	nd	$0.72^{f} \pm 0.01$	$0.03^{g} \pm 0.00$	$0.73^{c} \pm 0.02$	$0.04^{ed} \pm 0.00$	$11.58^{e} \pm 0.20$	$1.05^{c} \pm 0.02$	$68.16^{d} \pm 0.43$
3	$0.74^{f} \pm 0.02$	$53.18^{b} \pm 0.19$	$1.16^{de} \pm 0.08$	$0.03^{a} \pm 0.00$	$0.73^{f} \pm 0.02$	$0.04^{g} \pm 0.02$	$0.86^{b} \pm 0.02$	$0.05^{ed} \pm 0.02$	$12.53^{de} \pm 0.82$	$0.72^{e} \pm 0.02$	$70.04^{d} \pm 0.93$
Ca/Mg = 1:10											
0	$1.32^{e} \pm 0.01$	$19.23^{e} \pm 0.17$	$0.44^{fg} \pm 0.00$	nd	$2.28^{c} \pm 0.01$	$0.68^{d} \pm 0.03$	$0.43^{e} \pm 0.01$	$0.05^{ed} \pm 0.01$	$1.32^{h} \pm 0.00$	$0.31^{h} \pm 0.00$	$26.06^{h} \pm 0.16$
1	$1.35^{e} \pm 0.00$	$30.23^{d} \pm 0.06$	$0.97^{e} \pm 0.00$	nd	$2.39^{c} \pm 0.00$	$0.69^{d} \pm 0.07$	$1.26^{a} \pm 0.02$	$0.03^{ed} \pm 0.02$	$1.67^{g} \pm 0.03$	$0.47^{f} \pm 0.00$	$39.06^{g} \pm 0.04$
2	$2.04^{d} \pm 0.02$	$51.15^{b} \pm 0.06$	$1.23^{de} \pm 0.01$	nd	$4.46^{a} \pm 0.01$	$0.29^{f} \pm 0.02$	$0.55^{d} \pm 0.01$	$0.05^{ed} \pm 0.01$	$1.77^{g} \pm 0.04$	$0.22^{i} \pm 0.01$	$61.76^{e} \pm 0.07$
3	$2.38^{d} \pm 0.02$	$39.20^{c} \pm 0.35$	$1.79^{d} \pm 0.01$	nd	$3.20^{b} \pm 0.03$	$1.09^{a} \pm 0.02$	$0.24^{h} \pm 0.00$	$0.04^{ed} \pm 0.01$	$1.09^{h} \pm 0.01$	$0.68^{e} \pm 0.01$	$49.71^{f} \pm 0.04$

Identical superscripts denote no significant differences between mean for the fixed effect of Cu addition level and Ca/Mg ratio in two-way analysis of variance according to a post hoc HSD Tukey's test

Table 5 Pearson's correlation coefficient (r) for the linear relationship between analysed biological active compounds of *Salix purpurea* × *viminalis* and copper concentration in medium

Biological active compounds	Ca/Mg ratio			
	1:$^1/_4$	4:1	20:1	10:1
Total LMWOAs	0.9570	0.8745	0.8430	0.7948
Oxalic acid	−0.6862	0.8724	−0.0581	−0.2453
Total phenolics	0.9113	0.9491	0.9664	0.8637
Salicylic acid	0.7704	0.9489	0.9964	0.8954
Glucose	0.9190	0.9568	0.9836	0.9592
Fructose	0.9935	0.9058	0.9601	0.9942
Sucrose	0.9968	0.9912	0.9533	0.9807
Glutathione	0.6516	0.7739	0.6398	0.5303

every tested combination. The results showed that plants were able to release, in a short period of time, relatively high amounts of LMWOAs (Table 4).

For every combination, regardless of the Ca/Mg ratio, the total amount of analysed LMWOAs increased with increasing copper concentration in solution (Table 4). However, the strongest linear correlation was observed for 1:$^1/_4$ ($r = 0.9570$) and 4:1 ($r = 0.8745$) Ca/Mg ratios (Table 5).

The total concentration of LMWOAs under different Cu levels showed wide variation and increased from 38.29 to 226.85 (Ca/Mg = 4:1), from 27.89 to 135.47 (Ca/Mg = 20:1), from 40.92 to 70.04 (Ca/Mg = 1:$^1/_4$) and from 26.06 to 61.76 (Ca/Mg = 1:10) μM kg^{-1} dry weight (DW) of rhizosphere sample (Table 4). For a physiological level of Ca and Mg (4:1) oxalic and acetic organic acids were dominant, then succinic, citric, formic, lactic, malonic and malic acids. Oxalic acid concentration was strongly correlated with Cu level in the medium (Tables 4, 5).

Dependently of Ca/Mg ratio, malic, malonic, maleic and fumaric acids were detected at much lower levels (Table 4). In the case of 20:1 ratio, the main acids were formic and citric, then lactic, succinic, acetic, maleic and malic. The lowest value was observed for oxalic and malonic acids. For 1:$^1/_4$ Ca/Mg ratio the dominant acids were citric and oxalic and for 1:10 Ca/Mg citric acid. The other acids were at much lower concentrations, while fumaric acid was generally under the detection limit.

Total phenolics and salicylic acid content

The total phenolic content in leaves of *Salix* in a physiological Ca/Mg ratio (4:1) was ~14 mg GAE g^{-1} DW (control). The deficiency of two macroelements (Ca/Mg = 1: $^1/_4$) did not affect the total phenolics content, while the excess of Ca and Mg (20:1 and 1:10) caused the increase of their biosynthesis and accumulation in *Salix* leaves. The phenolics contents were significantly elevated with Cu level in the medium for all Ca/Mg ratios. The highest total phenolics content was recorded for the Ca/Mg ratio 20:1 and 3 mM Cu (Fig. 1a).

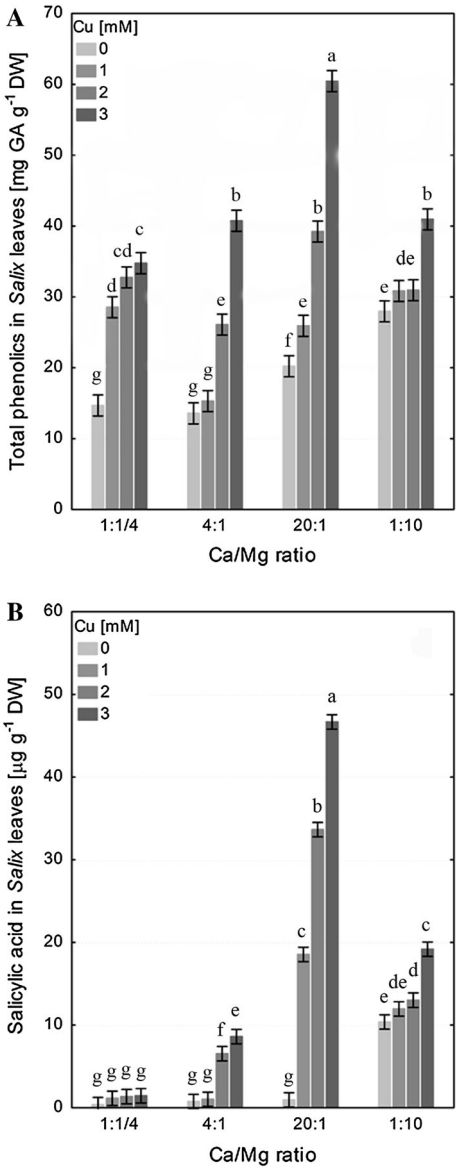

Fig. 1 Total phenolics (**a**) and salicylic acid (**b**) contents in leaves of Cu-treated *Salix Salix purpurea* × *viminalis* plants cultivated at different Ca/Mg ratios—mean values ($n = 6$; *vertical bars* show 95 % confidence interval for the fixed effect of Cu concentration and Ca/Mg ratio in two-way analysis of variance)

The Ca/Mg ratio significantly influenced the effect of copper concentration in the medium on the biosynthesis and accumulation of salicylic acid in *Salix* leaves. At the physiological level (Ca/Mg = 4:1), higher Cu treatments (2 and 3 mM Cu) caused the increase of SA content from ~1 up to 8 and 9 μg g^{-1} DW, respectively. The deficit of both macroelements, although applied at the same molar

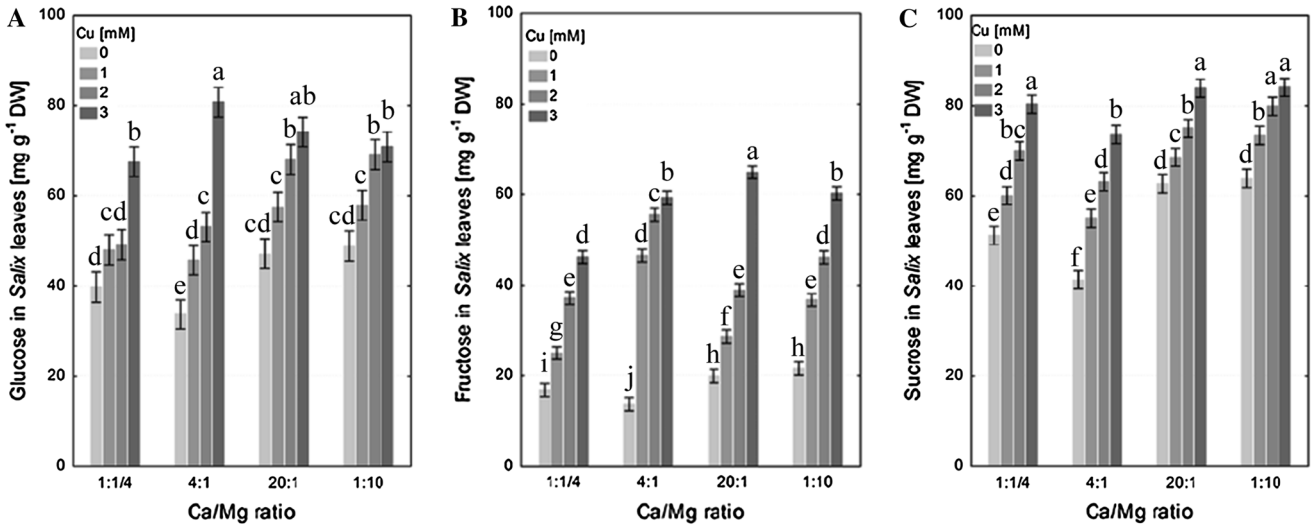

Fig. 2 Glucose (**a**), fructose (**b**) and sucrose (**c**) contents in leaves of Cu-treated *Salix Salix purpurea* × *viminalis* plants cultivated at different Ca/Mg ratios—mean values ($n = 6$; *vertical bars* show 95 % confidence interval for the fixed effect of Cu concentration and Ca/Mg ratio in two-way analysis of variance)

ratio as the physiological one ($1:^1/_4$), maintained the SA content at a lower and stable level in the case of all Cu treatments ($\sim$0.5 to 1 μg g^{-1} DW). The excess of calcium (Ca/Mg = 20:1) led to the strongest induction of SA biosynthesis for successive Cu levels (from $\sim$1 to 18, 34 and 47 μg g^{-1} DW at 1, 2 and 3 mM Cu, respectively). In contrast, with an excess of magnesium (Ca/Mg = 1:10), a reduction of the effect of Cu concentration in the medium on the SA content was observed (up to $\sim$19 μg g^{-1} DW at 3 mM Cu). However, the SA level in Cu-untreated plants was $\sim$10 times higher than in control plants cultivated at the other Ca/Mg ratios ($\sim$10 μg g^{-1} DW) (Fig. 1b).

Total phenolics and salicylic acid showed a stronger linear correlation with copper concentration in the medium in the case of Ca excess (Ca/Mg = 20:1), and slightly weaker at the physiological ratio (4:1) (Table 5).

Soluble carbohydrate content

The deficiency and excess of Ca and/or Mg caused an increase of glucose, fructose and sucrose concentrations in comparison to the physiological ratio of Ca and Mg (4:1). Additionally, Cu treatment at all Ca/Mg ratios resulted in significant accumulation of all soluble carbohydrates in *Salix* leaves (Fig. 2a–c). The increase of soluble sugars was almost linearly correlated with Cu level in the medium in the case of all investigated ratios of Ca and Mg (Table 5).

Thiols

In comparison with control Ca/Mg ratio (4:1), an excess of Mg (1:10) caused an increase of total GSH content, while a deficiency of Ca and Mg ($1:^1/_4$) caused a decrease in content of this thiol (Fig. 3).

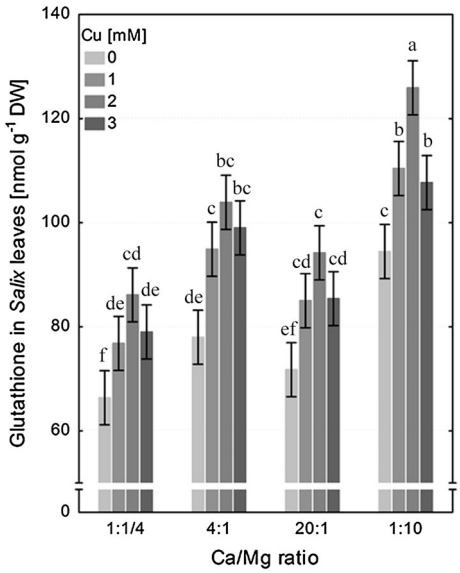

Fig. 3 Total glutathione content in leaves of Cu-treated *Salix Salix purpurea* × *viminalis* plants cultivated at different Ca/Mg ratios—mean values ($n = 6$; *vertical bars* show 95 % confidence interval for the fixed effect of Cu concentration and Ca/Mg ratio in two-way analysis of variance)

The addition of Cu ions to the medium intensified biosynthesis of GSH, with the simultaneous lack of detection of PCs, of which GSH is a precursor. The content of total GSH increased significantly with increasing Cu concentration for all Ca/Mg ratios, but the strongest linear correlation was observed at Ca/Mg = 4:1 (Table 5).

Discussion

The literature describes the interactions between some heavy metals in the sorption process in plants, as well as the relationship between metals in terms of their bioavailability (Ait Ali et al. 2004; Yanqun et al. 2004). There are also interactions in plants during the phytoremediation process (Kamnev and Van Der Lelie 2000). The aim of this study was to evaluate the effect of calcium and magnesium ions on *Salix* phytoremediation properties and biological active compounds of the plant response to elevated Cu concentrations. Results presented efficiency of Cu accumulation in *Salix purpurea* × *viminalis* roots, shoots, bark, rods and leaves with information about changes in willow biomass were presented in Part 1. (Mleczek et al. 2013). On the basis of biometric determinations it was found that the ratio of Ca to Mg significantly modified the bioaccumulation of Cu and willow growth. Efficiency of Cu accumulation declined according to the trend: 1:10 > 4:1 > 20:1 > $1:^{1}/_{4}$ (Mleczek et al. 2012). A similar relationship was observed in the earlier studies on Cd and Pb accumulation (Chadzinikolau et al. 2011), or Cu and Zn accumulation (Mleczek et al. 2012) by *Salix*. There are only a few literature data concerning this issue. High concentrations of magnesium and calcium decreased nickel hyperaccumulation in *Alyssum bertolonii* (Vergnano Gambi et al. 1992). In another hyperaccumulator, *Silene armeria*, the effect of the Ca to Mg ratio on copper accumulation and the tolerance towards this element was dependent on the adaptation of ecotypes to soil conditions, including the calcium content in the soil. Accumulation of toxic metals can be modulated by blocking them within the cell wall and outer plasmalemma surface. In this regard, calcium ions may be more effective than magnesium ions (Lombini et al. 2003). On account of the tight control of calcium ion transport between the apoplast and symplast, plants are tolerant of high extracellular concentrations of this cation. This element may therefore affect the extracellular accumulation of heavy metals along with the intensity of elongation growth of plants.

Four Ca/Mg ratios were used to indicate the differences in LMWOA exudation into the *Salix* root zone under increasing Cu concentrations, i.e. the physiological ratio (4:1), excess calcium (20:1), excess magnesium (1:10), and a deficit of both macronutrients ($1:^{1}/_{4}$). Such combinations were chosen because the composition of the cultivation medium, including Ca and Mg ions, might be crucial for the potential alleviation of copper toxicity to plants used in phytoremediation/recultivation (presumably via competition for cell-surface binding sites) (Karamushka and Gadd 1994). Previous studies have shown that higher concentrations of Ca or Mg, as well as changes in the Ca/Mg ratio, alleviated phytotoxicity of Mn (Parker et al. 1998), Ni (Gabrielli and Pandolfini 1984; Parker et al. 1998), Pb (Jowett 1964; Parker et al. 1998), and Zn (Simon and Lefèbvre 1977; Parker et al. 1998). Quantitative studies of the impact of either Ca or Mg on Cu toxicity in higher plants are still limited.

Plants actively modify conditions in the rhizosphere by changes in redox conditions (Jacob and Otte 2004a, b; Mucha et al. 2005), mineral precipitation/dissolution (Mucha et al. 2010), and exudation of organic substances capable of complexing metals and changing their bioavailability (Parker et al. 2001; Hammer and Keller 2002; Berkelaar and Hale 2003; Magdziak et al. 2011). Root-exuded LMWOAs influence the behaviour of nutrients and trace metals, either enhancing or reducing their availability, by directly affecting acidification, chelation, precipitation and redox reactions, or indirectly, through their effects on microbial activity, physical and chemical properties of the rhizosphere and root growth pattern (Tao et al. 2004; Kidd et al. 2009; Li et al. 2009). Recent studies have shown that *Salix viminalis* L. can release to the root zone relatively high amounts of LMWOAs (Magdziak et al. 2011), and simultaneously exhibits moderate Cu uptake to the aboveground organs (Gąsecka et al. 2012). For these reasons, LMWOAs are considered efficient either to increase the bioavailability/mobility and the rate of Cu uptake sorption by *Salix Salix purpurea* × *viminalis*, or to serve as a first step of defence against toxic copper concentration through complexation with soil matrix, or direct transport of less toxic forms to the cell vacuole. Studies carried out in a hydroponic system with *Salix* have demonstrated that the release of LMWOAs by roots is not only a response to increasing Cu concentration. It was also observed that the release of organic acids from roots significantly differed among the Ca/Mg ratios. In the case of the 4:1 Ca/Mg ratio, a physiological one, the induction of root exudation of oxalic and acetic acids was strongly induced by copper ions. The exudation of these acids may be the reaction of the plant to the oxidative stress induced by metallic ions, which activated mechanisms involving the tolerance to toxic elements, i.e. chelation and further transport via xylem followed by immobilization in the vacuole (Nigam et al. 2001). In the case of the 20:1 Ca/Mg ratio, the dominant acids were citric and formic, for $1:^{1}/_{4}$ citric and oxalic acids, and for 1:10 they were citric and lactic acids. In the case of these combinations citric acid is the main acid that has been most often reported (next to malonate) in plant exudates (Jones 1998). Since citric and lactic acids efficiently bind Ca and Mg ions, their exudation by *Salix* roots is probably the result of the plant reaction to a modified concentration (and molar ratio) of macroelements in the cultivation medium (Magdziak et al. 2011). High concentrations of LMWOAs decreased the pH of the cultivation medium, and solubility and mobility Cu, which

influenced reduction of the element's adsorption by structural elements of the sand and increased the concentrations of soluble forms and bioavailability of copper to the plant (Harter 1983). However, the composition of organic acids exuded into the rhizosphere is crucial for the uptake capacity, due to the differences in their binding capacity towards selected metals (Qin et al. 2007; Mucha et al. 2010).

Chelation of metals by high-affinity ligands such as LMWOAs, amino acids or phytochelatins (PCs) is a very important mechanism of heavy metal detoxification (Clemens 2001; Freeman et al. 2004). We did not observe the formation of PCs in leaves of *Salix purpurea* × *viminalis*, although these molecules may play a role in Cu stress tolerance (Cobbett and Goldsbrough 2002; Yruela 2005). Similar results were reported in our earlier studies (Gąsecka et al. 2012), as well as for various clones of this species, with different metal tolerance (Landberg and Greger 2004). The results of the present study reveal that increasing Cu concentration in the medium stimulated an increase in levels of GSH, which might serve not only as an important antioxidant, but also as a metal chelator (Yadav 2010). Unfortunately, the elevation of GSH does not always correlate with enhanced tolerance to metals (Xiang et al. 2001). The GSH content depends on sources and level of metals, plant species or plant organs studied, and also on experimental conditions, mainly on the presence of additional stressors (Anjum et al. 2012). In our studies, total GSH level was also dependent on the ratio of calcium and magnesium in the medium.

The experiment showed that excess of the macroelements elevated the total phenolics content in *Salix* leaves. The induction of their biosynthesis confirmed their protective role under stress conditions. As in our earlier experiments (Drzewiecka et al. 2012a; Gąsecka et al. 2012) we observed a Cu- and Ni-induced increase in the content of phenolics, which probably act as metal chelators, reactive oxygen species (ROS) scavengers and antioxidants (Dixon and Paiva 1995; Michalak 2006). Additionally, Cu was confirmed to stimulate the peroxidase enzymes under ROS stress which induce lignification (Halliwell 1978). Because of that we can suppose that phenols could play as ROS scavengers during oxidative stress induced by metals.

In the present study, the strongest accumulation of phenolics was observed in the case of 20:1 Ca/Mg ratio and 3 mM Cu, suggesting excessive phytotoxicity of the metal under excess of calcium and deficit of magnesium. Calcium, magnesium and copper participate in regular plant development and both their deficiency and excess have an impact on the metabolism. Although *Salix* is a plant that readily adapts to new environmental conditions (Hermle et al. 2006), we postulate that irregular Ca/Mg ratios influence Cu sorption and phytotoxicity. Among phenolic compounds synthesized in plant cells, salicylic acid in particular plays an important role in plant defence against external factors causing the phenomenon of oxidative stress (Dixon and Paiva 1995). Salicylic acid content in plants has been reported to reach even ~ 75 μg g^{-1} DW when plants are challenged with acute viral infection (e.g. tobacco mosaic virus). However, abiotic stressors may also induce the enhanced biosynthesis of salicylic acid, which is part of the controlled intracellular response that mimics the hypersensitive response to pathogens. As previously described (Drzewiecka et al. 2012b), salicylic acid is postulated as a plant biomarker of oxidative stress often strongly correlated with the stressor intensity. In the present study, the highest salicylic acid biosynthesis was induced in plants cultivated at Ca/Mg = 20:1, i.e. with excess calcium in the cultivation medium. This suggests that at this ratio, the defence reaction to copper was the strongest and probably resulted from the enhanced transport of Cu to the cell interior, or Cu-caused disturbance of calcium channels within the cell membrane. As previously reported, calcium intensified the toxic effect of copper ions to bean plants (Maksymiec and Baszyński 1999). This confirms diminished induction of salicylic acid biosynthesis in *Salix* plants cultivated with a calcium deficit.

The disturbances in physiological Ca/Mg ratio caused an increase in glucose, fructose and sucrose contents in *Salix* leaves. Moreover, their concentrations were also elevated with the Cu level in the medium. The results confirmed our earlier studies concerning the influence of metals on accumulation of photoassimilates in leaves (Drzewiecka et al. 2012a; Gąsecka et al. 2012). In regular plant metabolism assimilates are produced in leaves, then translocated and stored in different tissues in response to sink/source relations regulated by partitioning of starch and sucrose biosynthesis (Taiz and Zeiger 2006). The accumulation of soluble carbohydrates in leaves probably results from disturbances in starch hydrolysis. The consequences of the accumulation of assimilates, as documented previously, are changes in the rate of photosynthesis and disturbances in the sink/source balance within a plant, leading to premature senescence (Wingler et al. 2006). In several studies, sugars were postulated to be signalling molecules controlling gene expression and developmental processes in plants (Sheen et al. 1999; Morkunas et al. 2005). Based on our previous results (Drzewiecka et al. 2012a; Gąsecka et al. 2012) we suggest that soluble sugars can also play a signalling role in defence mechanisms in plants cultivated in metal-polluted media. Additionally, an excessive accumulation of sugars could help the plant to bind metals and entrap them into cellulose structures within the cell wall, so the high quantity of photosynthesis products can be related to a detoxification activity as well.

Conclusions

The results obtained in the hydroponic system showed that Cu concentration and Ca/Mg ratio have a significant impact on the LMWOAs exuded by roots. The results confirm that the main role of LMWOAs is their complexing ability, which is crucial for mobilization of trace elements and metallic macronutrients, as well as metal detoxification, which constitutes an important element of the phytoremediation process. The results indicated that the deficiency and excess of Ca and/or Mg caused the accumulation of soluble carbohydrates, phenolic compounds and glutathione in *Salix* leaves. Moreover, the Cu treatments also influenced these concentrations at all Cu/Mg ratios. The changes in sugar contents probably result from disturbances in starch hydrolysis and sugar transport. The disturbances negatively affect the rate of photosynthesis. The induction of phenolics biosynthesis under an irregular Ca/Mg ratio had an impact on Cu sorption and phytotoxicity. Simultaneously, biosynthesis of salicylic acid (a biomarker of the oxidative stress caused by copper) following the Cu treatments was enhanced in the conditions of calcium excess, and strongly reduced by a deficit of this macroelement.

Authors contribution Kinga Drzewiecka—statistical analysis, manuscript preparation. Mirosław Mleczek—corresponding author, manuscript preparation, experiment preparation. Monika Gąsecka—plant material preparation, sugars and phenols analysis, experiment preparation. Tamara Chadzinikolau—thiols analysis. Zuzanna Magdziak—low molecular weight organic acids analysis, experiment preparation. Piotr Goliński—manuscript preparation.

Acknowledgments The experimental part of this study was supported by the Ministry of Science and Higher Education, Grant No. N R12 0065 10.

References

Ait Ali N, Pilar Bernal M, Ater M (2004) Tolerance and bioaccumulation of cadmium by *Phragmites australis* grown in the presence of elevated concentrations of cadmium, copper and zinc. Aquat Bot 80:163–176. doi:10.1016/j.aquabot.2004.08.008

Albarracín V, Amoroso MJ, Abate CM (2010) Bioaugmentation of copper polluted soil microcosms with *Amycolatopsis tucumanensis* to diminish phytoavailable copper for *Zea mays* plants. Chemosphere 79:131–137. doi:10.1016/j.chemosphere.2010.01.038

Anjum NA, Ahmada I, Mohmood I, Pacheco M, Duarte AC, Pereira E, Umar S, Ahmad A, Khan NA, Iqbal M, Prasad MNV (2012) Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. Environ Exp Bot 75:307–324. doi:10.1016/j.envexpbot.2011.07.002

Barabasz A, Krämer U, Hanikenne M, Rudzka J, Antosiewicz DM (2010) Metal accumulation in tobacco expressing *Arabidopsis halleri* metal hyperaccumulation gene depends on external supply. J Exp Bot 61:3057–3067. doi:10.1093/jxb/erq129

Berkelaar E, Hale BA (2003) Accumulation of cadmium by durum wheat roots: bases for citrate-mediated exceptions to the free ion model. Environ Toxicol Chem 22:1155–1161. doi:10.1002/etc.5620220526

Chadzinikolau T, Kozłowska M, Mleczek M (2011) Calcium and magnesium influence on phytoremediation of heavy metals using *Salix viminalis* L. Nauka Przyroda Technologie 5(6):1–10

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212:475–486. doi:10.1007/s004250000458

Cobbett CS, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Physiol 53:159–182. doi:10.1146/annurev.arplant.53.100301.135154

Dixon R, Paiva N (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097. doi:10.1105/tpc.7.7.1085

Drzewiecka K, Mleczek M, Gąsecka M, Magdziak Z, Goliński P (2012a) Changes in *Salix viminalis* L. cv. 'Cannabina' morphology and physiology in response to nickel ions—hydroponic investigations. J Hazard Mater 217–218:429–438. doi:10.1016/j.jhazmat.2012.03.056

Drzewiecka K, Mleczek M, Waśkiewicz A, Goliński P (2012b) Oxidative stress and phytoremediation. In: Parvaiz A, Prasad MNV (eds) Abiotic stress responses in plants: metabolism, productivity and sustainability. Springer, pp 425–451

Elguindi J, Hao X, Lin Y, Alwathnani HA, Wei G, Rensing C (2011) Advantages and challenges of increased antimicrobial copper use and copper mining. Appl Microbiol Biotechnol 91:237–249. doi:10.1007/s00253-011-3383-3

Freeman JL, Persans MW, Nieman K, Albrecht C, Peer W, Pickering IJ, Salt DE (2004) Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. Plant Cell 16:2176–2191. doi:10.1105/tpc.104.023036

Gabrielli R, Pandolfini T (1984) Effect of Mg^{2+} and Ca^{2+} on the response to nickel toxicity in a serpentine endemic and nickel-accumulating species. Plant Physiol 62:540–544. doi:10.1111/j.1399-3054.1984.tb02796.x

Gąsecka M, Mleczek M, Drzewiecka K, Magdziak Z, Rissmann I, Chadzinikolau T, Golinski P (2012) Physiological and morphological changes in *Salix viminalis* L. as a result of plant exposure to copper. J Environ Sci Health A 74:33–40. doi:10.1080/10934529.2012.650557

Halliwell B (1978) Lignin synthesis: the generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta 140:81–88

Hammer D, Keller C (2002) Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractants. J Environ Qual 31:1561–1569. doi:10.2134/jeq2002.1561

Harter RD (1983) Effect of soil pH on adsorption of lead, copper, zinc and nickel. Soil Sci Soc Am J 47:47–51

Hermle S, Günthardt-Goerg MS, Schulin R (2006) Effects of metal contaminated soil on the performance of young trees growing in model ecosystems under field conditions. Environ Pollut 144:703–714. doi:10.1016/j.envpol.2005.12.040

Jacob DL, Otte ML (2004a) Long-term effects of submergence and wetland vegetation on metals in a 90-year old abandoned Pb-Zn

mine tailings pond. Environ Pollut 130:337–345. doi:10.1016/j.envpol.2004.01.006

Jacob DL, Otte ML (2004b) Influence of *Typha latifolia* and fertilization on metal mobility in two different Pb-Zn mine tailing types. Sci Total Environ 333:9–24. doi:10.1016/j.scitotenv.2004.05.005

Jones DL (1998) Organic acids in the rhizosphere—a critical review. Plant Soil 205:25–44. doi:10.1023/A:1004356007312

Jowett D (1964) Population studies on lead-tolerant *Agrostis tenuis*. Evolution 18:70–81

Kabata-Pendias A, Pendias H (1999) Biogeochemia pierwiastków śladowych. Biogeochemistry of trace elements, 3rd edn. Wyd, Naukowe PWN, Warsaw (in Polish)

Kamnev AA, Van Der Lelie D (2000) Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. Biosci Rep 20:239–258. doi:10.1023/A:1026436806319

Karamushka VI, Gadd GM (1994) Interaction of *Saccharomyces cerevisiae* with gold: toxicity and accumulation. Biometals 12:289–294. doi:10.1023/A:1009210101628

Kidd P, Barcelo J, Pilar Bernal M, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R, Monterroso C (2009) Trace element behaviour at the root–soil interface: implications in phytoremediation. Environ Exp Bot 67:243–259. doi:10.1016/j.envexpbot.2009.06.013

Landberg T, Greger M (2004) No phytochelatin (PC2 and PC3) detected in *Salix viminalis*. Physiol Plant 121:481–487. doi:10.1111/j.0031-9317.2004.00347.x

Lewandowski I, Schmidt U, Londo M, Faaij A (2006) The economic value of the phytoremediation function—assessed by the example of cadmium remediation by willow (*Salix* ssp). Agric Syst 89:68–89. doi:10.1016/j.agsy.2005.08.004

Li JT, Liao B, Dai ZY, Zhu R, Shu WS (2009) Phytoextraction of Cd-contaminated soil by carambola (*Averrhoa carambola*) in field trials. Chemosphere 76:1233–1239. doi:10.1016/j.chemosphere.2009.05.042

Lombini A, Llugany M, Poschenrieder CH, Dinelli E, Barcelo J (2003) Influence of the Ca/Mg ratio on Cu resistance in three *Silene armeria* ecotypes adapted to calcareous soil or to different, Ni- or Cu-enriched, serpentine sites. J Plant Physiol 160:1451–1456. doi:10.1078/0176-1617-01002

Magdziak Z, Kozlowska M, Kaczmarek Z, Mleczek M, Chadzinikolau T, Drzewiecka K, Golinski P (2011) Influence of Ca/Mg ratio on phytoextraction properties of *Salix viminalis*. II. Secretion of low molecular weight organic acids to the rhizosphere. Ecotoxicol Environ Saf 74:33–40. doi:10.1016/j.ecoenv.2010.09.003

Maksymiec W, Baszyński T (1999) Are calcium ions and calcium channels involved in the mechanisms of Cu^{2+} toxicity in bean plants? The influence of leaf age. Photosynthetica 36:267–278. doi:10.1023/A:1007007929102

Michalak A (2006) Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish J Environ Stud 15:523–530

Mleczek M, Kozłowska M, Kaczmarek Z, Chadzinikolau T, Goliński P (2012) Influence of Ca/Mg ratio on phytoextraction properties of *Salix viminalis*. I. The effectiveness of Cd, Cu, Pb, and Zn bioaccumulation and plant growth. Int J Phytoremed 14:75–88. doi:10.1080/15226514.2011.573824

Mleczek M, Gąsecka M, Drzewiecka K, Goliński P, Magdziak Z, Chadzinikolau T (2013) Copper phytoextraction with willow (*Salix viminalis* L.) under various Ca/Mg ratios. Part 1. Copper accumulation and plant morphology changes. Acta Physiol Plant 35:3251–3259. doi:10.1007/s11738-013-1360-4

Morkunas I, Marczak Ł, Stachowiak J, Stobiecki M (2005) Sucrose-induced lupine defense against *Fusarium oxysporum*: sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*. Plant Physiol Biochem 43:363–373. doi:10.1016/j.plaphy.2005.02.011

Mucha AP, Almeida CMR, Bordalo AA, Vasconcelos MTSD (2005) Exudation of organic acids by a marsh plant and implications on trace metal availability in the rhizosphere of estuarine sediments. Estuar Coast Shelf Sci 65:191–198. doi:10.1016/j.ecss.2005.06.007

Mucha AP, Almeida CMR, Bordalo AA, Vasconcelos MTSD (2010) LMWOA (low molecular weight organic acid) exudation by salt marsh plants: natural variation and response to Cu contamination. Estuar Coast Shelf Sci 88:63–70. doi:10.1016/j.ecss.2010.03.008

Najeeb U, Xu L, Ali S, Jilani G, Gong HJ, Shen WQ, Zhou WJ (2009) Citric acid enhances the phytoextraction of manganese and plant growth by alleviating the ultrastructural damages in *Juncus effusus* L. J Hazard Mater 170:1156–1163. doi:10.1016/j.jhazmat.2009.05.084

Nigam R, Srivastava S, Prakash S, Srivastava MM (2001) Cadmium mobilization and plant availability—the impact of organic acid commonly exuded from roots. Plant Soil 230:107–113. doi:10.1023/A:1004865811529

Ösretås AH, Greger M (2006) Interactions between calcium and copper or cadmium in Norway spruce. Biol Plant 50:647–652. doi:10.1007/s10535-006-0101-6

Oze C, Skinner C, Schroth AW, Coleman RG (2008) Growing up green on serpentine soils: biogeochemistry of serpentine vegetation in the Central Coast Range of California. Appl Geochem 23:3391–3403. doi:10.1016/j.apgeochem.2008.07.014

Parker DR, Pedler JF, Thomason DN, Li H (1998) Alleviation of copper rhizotoxicity by calcium and magnesium at defined free metal-ion activities. Soil Sci Soc Am J 6:965–972. doi:IND43627815

Parker DR, Pedler JF, Ahnstrom ZAS, Resketo M (2001) Reevaluating the free-ion activity model of trace metal toxicity toward higher plants: experimental evidence with copper and zinc. Environ Toxicol Chem 20:899–906. doi:10.1023/A:1004249923989

Peñarrubia L, Andrés-Colás N, Moreno J, Puig S (2010) Regulation of copper transport in *Arabidopsis thaliana*: a biochemical oscillator? J Biol Inorg Chem 15:29–36. doi:10.1007/s00775-009-0591-8

Qin R, Hirano Y, Brunner I (2007) Exudation of organic acid anions from poplar roots after exposure to Al, Cu and Zn. Tree Physiol 27:313–320. doi:10.1093/treephys/27.2.313

Scrase-Field SAMG, Knight MR (2003) Calcium: just a chemical switch? Curr Opin Plant Biol 6:500–506. doi:10.1016/S1369-5266(03)00091-8

Sheen J, Zhou L, Jang J-Ch (1999) Sugars as signaling molecules. Curr Opin Plant Biol 2:410–418

Simon E, Lefèbvre C (1977) Aspects de la tolerance aux metaux lourds chez *Agrostis tenuis* Sibth., *Festuca ovina* L. et *Armeria maritima* (Mill.) Willd. Acta Oecolo Oecol Plant 12:95–100

Stolarski M, Szczukowski S, Tworkowski J, Klasa A (2008) Productivity of seven clones of willow coppice in annual and quadrennial cutting cycles. Biomass Bioenerg 32:1227–1234. doi:10.1016/j.biombioe.2008.02.023

Strobel BW (2001) Influence of vegetation on low-molecular-weight carboxylic acids in soil solution-a review. Geoderma 99:169–198. doi:10.1016/S0016-7061(00)00102-6

Stroiński A, Zielezińska M (1997) Cadmium effect on hydrogen peroxide, glutathione and phytochelatins levels in potato tuber.

Acta Physiol Plant 19:127–136. doi:10.1007/s11738-997-0029-2
Taiz RL, Zeiger E (2006) Plant physiology, 4th edn. Sinauer Associates, Sunderland
Tao S, Liu WX, Chen YJ, Xu FL, Dawson RW, Li BG, Cao J, Wang XJ, Hu JY, Fang JY (2004) Evaluation of factors influencing root-induced changes of copper fractionation in rhizosphere of a calcareous soil. Environ Pollut 129:5–12. doi:10.1016/j.envpol.2003.10.001
Tlustŏs P, Száková J, Vysloŭzilová M, Pavlíková D, Weger J, Javorská H (2007) Variation in the uptake of arsenic, cadmium, lead, and zinc by different species of willows *Salix* spp. grown in contaminated soils. Cent Eur J Biol 2:254–275. doi:10.2478/s11535-007-0012-3
Tukendorf A, Rauser WE (1990) Changes in glutathione and phytochelatins in roots of maize seedlings exposed to cadmium. Plant Sci 70:155–166. doi:10.1016/0168-9452(90)90129-C
Vergnano Gambi O, Gabbrielli R, Pandolfini T (1992) Some aspects of the metabolism of *Alyssum bertolonii* Desv. In: Baker AJM, Proctor J, Reeves RD (eds) The vegetation of ultramafic (Serpentine) soils. Intercept, Andover, pp 319–329
White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92:487–511. doi:10.1093/aob/mcg164
Wingler A, Purdy S, MacLean A, Pourtau N (2006) The role of sugars in integrating environmental signals during the regulation of leaf senescence. J Exp Bot 57:391–399. doi:10.1093/jxb/eri279
Xiang C, Werner BL, Christensen EM, Oliver DJ (2001) The biological functions of glutathione revisited in *Arabidopsis* transgenic plants with altered glutathione levels. Plant Physiol 126:564–574. doi:10.1104/pp.126.2.564
Yadav SK (2010) Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S Afr J Bot 76:167–179. doi:10.1016/j.sajb.2009.10.007
Yanqun Z, Yuan L, Schvartz C, Langladec L, Fan L (2004) Accumulation of Pb, Cd, Cu and Zn in plants and hyperaccumulator choice in Lanping lead-zinc mine area, China. Environ Int 30:567–576. doi:10.1016/j.envint.2003.10.012
Yruela I (2005) Toxic metals in plants: copper in plants. Br J Plant Physiol 17:145–156. doi:10.1590/S1677-04202005000100012